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Transdominant Human T-Cell Lymphotropic Virus Type I TAX_1 Mutant That Fails To Localize to the Nucleus

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Human T-cell lymphotropic virus type I (HTLV-I) encodes a 40-kDa nuclear transactivating phosphoprotein, TAX_1. The results presented in this study demonstrate that deletion of amino acids 2 through 59 of TAX_1 (Δ58 TAX_1) decreased transactivation of the HTLV-I long terminal repeat 10- to 20-fold. S1 nuclease analysis revealed that the decrease in transactivation of the HTLV-I long terminal repeat was associated with a lack of RNA synthesis. In contrast to the nuclear localization of the wild-type TAX_1 protein, indirect immunofluorescence analysis demonstrated that Δ58 TAX_1 failed to localize to the nucleus, indicating that the TAX_1 nuclear localization sequence is present in amino acids 2 through 59. Cotransfection of wild-type and mutant TAX_1 DNAs resulted in the cytoplasmic accumulation of TAX_1 and a 25-fold decrease in transactivation. Although several possibilities which may account for this transdominant effect exist, we favor a model in which Δ58 TAX_1 interferes with the nuclear localization of wild-type TAX_1 protein, perhaps by forming heterodimer complexes.

Human T-cell lymphotropic virus type I (HTLV-I) is the causative agent of the lymphoproliferative disease adult T-cell leukemia/lymphoma (50, 69) and of the neurologic disease tropical spastic paraparesis/HTLV-I associated myelopathy (3, 4, 29). Recently, it has also been proposed that HTLV-I may be involved in the pathogenesis of Sjögren's syndrome (20). In addition to the *gag*, *pol*, and *env* genes, the viral genome of HTLV-I contains a unique region which encodes two transregulatory proteins, TAX_1 and REX_1, and a third protein, $p21^x$, whose function is unknown. TAX_1, a 40-kDa protein, is a positive transactivator of viral and cellular transcription (7, 10a, 12, 19, 27, 35, 39, 51, 53, 57, 62). REX_1 encodes a 27-kDa protein which functions posttranscriptionally by facilitating the cytoplasmic accumulation and transport of singly spliced and unspliced mRNAs that encode the viral structural proteins (22, 25, 28, 43). Both TAX_1 and REX_1 are nuclear phosphoproteins (10a, 15, 30, 60, 61) which are important for viral replication and have been shown to immortalize T cells when expressed in a herpesvirus saimiri vector (18). TAX_1 has also been shown to transform NIH 3T3 cells (64) and to cooperate with *RAS-1* to transform Rat-1 cells (46).

Deletion and site-specific mutagenesis are often used to delineate specific functional domains of regulatory gene products. For example, a wide range of deletion, point, and site-specific mutations in the simian virus 40 (SV40) T antigen have defined at least seven functional domains in this complex transforming and transcriptional regulatory protein (54). Similarly, site-directed mutagenesis of human immunodeficiency virus type 1 (HIV-1) *tat* demonstrated that the amino terminus and several cysteine amino acids were necessary for transactivation of the HIV-1 long terminal repeat (LTR) and that a 5-amino-acid sequence from the basic amino acid region of Tat acted as a nuclear localization signal sequence (52). Interestingly, the functionally inactive Tat mutant gene products were found to be inhibitory to the activity of the wild-type gene product when both genes were present together (21, 45). This transdominant inactivation has also been seen with specific mutations of Ha-ras (6, 63), HIV-1 Gag (66), HIV-1 Rev (38, 67), herpes simplex virus type 1 (HSV-1) VP16 (65), HSV-1 ICP4 (56), SV40 T antigen (34), adenovirus type 5 E1A (14), HTLV-I REX_1 (49), and HTLV-II TAX_2 (68). The discovery of transdominant inhibitors has contributed to the understanding of different domains within these proteins and raised the possibility that these mutants may be therapeutically useful in these viral infections.

During the characterization of the functional sequences of several nuclear proteins, specific amino acid sequences have been found to be responsible for targeting the protein for localization in the nucleus. The SV40 T antigen contains a sequence, Lys-Lys-Lys-Arg-Lys, which is important for nuclear localization (31, 32). For adenovirus E1A, the sequence Lys-Arg-Pro-Arg-Pro is important for nuclear localization (37). Although there is amino acid homology between the nuclear signal sequences of some of the proteins, the nuclear localization signals of other proteins are quite disparate. The most common aspect of these signaling sequences is the presence of highly basic amino acids. A possible interaction of these amino acid sequences at the nuclear envelope, specifically the nuclear pore structures within the nuclear envelope, has been suggested (10).

We have analyzed an amino-terminal deletion mutant of HTLV-I TAX_1 to evaluate the importance of this domain in transactivational activity. This mutant was transcriptionally inactive and had a transdominant inhibitory effect on the transactivational function of wild-type TAX_1. The cytoplasmic localization of the mutant protein suggests that the transdominant effect was due to inefficient transport of the wild-type TAX_1 protein to the nucleus of the cell.

MATERIALS AND METHODS

Cell culture, transfections, and CAT assays. African green monkey kidney cells (CV-1) were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 584 mg of L-glutamine per liter, 4,500 mg of D-glucose per liter, 100 U of penicillin G per ml, and 100 μg of streptomycin sulfate per ml at 37°C in a 5% CO_2 environ-

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ment. Transfections for chloramphenicol acetyltransferase (CAT) assays were performed in 35-mm plates beginning with 2×10^5 cells per plate as described previously (16, 17). Transfections for RNA analysis were performed on 100-mm dishes beginning with 1.6×10^6 cells per plate as above. Forty-eight hours after transfection, the cells were harvested and the cytoplasmic RNA was isolated as described below for S1 nuclease evaluation.

Plasmid constructions. Plasmids were constructed from the TAX_1 coding region from HTLV-I TAX_1, which has been previously described (41). Sequences encoding 58 of the first 59 amino acids of TAX_1 were deleted by removing the 186-bp *Hin*dIII-*Cla*I restriction fragment (Fig. 1A) and inserting a 20-bp double-stranded oligonucleotide (Fig. 1B), replacing the ATG initiation codon and keeping the remainder of the protein coding sequence in frame. This construct is referred to as HTLV-I Δ58 TAX_1. Proper construction of this plasmid was confirmed by restriction enzyme digestion analysis and by the dideoxynucleotide sequencing method of Sanger et al. (Sequenase kit; U.S. Biochemical Corp.) (55).

The wild-type and deletion mutant coding sequences of TAX_1 were also placed into a cytomegalovirus (CMV) promoter expression vector, pCMV-1 (2) (Fig. 1C). HTLV-I TAX_1 and HTLV-I Δ58 TAX_1 were digested at the *Bam*HI restriction site where a *Bam*HI-*Hin*dIII oligonucleotide linker sequence was ligated. Subsequently, the TAX_1 and Δ58 TAX_1 coding sequences were removed by digestion with *Hin*dIII. The coding sequences were then ligated into pCMV-1 at its unique *Hin*dIII site. Proper orientation of the coding sequence was confirmed by restriction enzyme digestion analysis and by nucleotide sequencing according to the Sequenase (U.S. Biochemical) dideoxynucleotide method of Sanger et al. (55).

^{35}S metabolic labeling of transfected cells and immunoprecipitation assay. Transfections of CV-1 cells were performed, as described above, in 35-mm tissue culture plates. Forty-four hours after transfection, the cells were radiolabeled and immunoprecipitations were performed according to the procedure of Harlow and Lane (23). Briefly, the cells were radiolabeled with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN Biomedicals, Inc.) in methionine-free Dulbecco's modified Eagle's medium with 10% dialyzed fetal calf serum, 584 mg of L-glutamine per liter, 4,500 mg of D-glucose per liter, 100 U of penicillin G per ml, and 100 μg of streptomycin sulfate per ml for 4 h. The metabolically radiolabeled cells were lysed in 1 ml of RIPA buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 μg of aprotinin per ml) for 30 min on ice. The lysed cell supernatant was incubated with 50 μl of normal rabbit serum for 30 min on ice and then incubated twice (30 min each time) and cleared of the supernatant with 50 μl of prepared, clean 10% Pansorbin (Calbiochem). The remaining supernatant was divided into two samples which had 50 μl of either rabbit preimmune serum or polyclonal anti-TAX_1 serum per ml and incubated overnight (12 to 18 h) on ice. Moist 10% Pansorbin (50 μl) was added and incubated for 60 min with gentle rocking at 4°C. The Pansorbin-antibody complex was pelleted and washed four times with cold (4°C) RIPA buffer. The Pansorbin-antibody pellet was then resuspended in 50 μl of 2× SDS-denaturation buffer (0.125 M Tris base, 69 mM SDS, 20% glycerol, 1% bromophenol blue; pH 6.90) with 5% 2-mercaptoethanol and heated at 90 to 100°C for 10 min before electrophoresis in an SDS–10% polyacrylamide gel (SDS-PAGE). The gel was fixed in 40% methanol–10% acetic acid for 15 min, soaked in Enlightning (NEN-Du Pont) for 30 min, dried on Whatman 3MM filter paper, and exposed to X-ray film.

As a control, 10^7 C81 cells (an HTLV-I-transformed cell line) were radiolabeled and immunoprecipitated as described above.

Indirect immunofluorescence. CV-1 cells plated on sterile glass microscope coverslips in 35-mm dishes were transfected with wild-type or mutant TAX_1, as described above. Forty-eight hours after transfection, the cells were washed three times with cold (4°C) phosphate-buffered saline (PBS) without calcium or magnesium and allowed to air dry. The cells were then fixed onto the coverslip by submersion in acetone-methanol (2:1) at −20°C for 10 min and then air dried. Fixed coverslips were stored in parafilmed dishes at −20°C until staining.

One-quarter of the fixed coverslip was prepared for staining by moistening with PBS for 5 to 10 min at room temperature. Approximately 20 μl of normal swine serum was added to cover the entire coverslip. After 30 min at room temperature, the coverslip was washed five times with 2 ml of PBS (the last wash for 5 min). Approximately 20 μl of rabbit polyclonal anti-TAX_1 serum was then added to cover each coverslip and allowed to react for 30 min at room temperature. After five washes with 2 ml of PBS, 20 μl of rhodamine isothiocyanate-conjugated swine anti-rabbit serum was added to the coverslip. After 30 min, the coverslips were washed with five 2-ml PBS washes (the last wash was for 15 min). Stained coverslips were mounted onto glass microscope slides and stored at 4°C until viewed with a UV fluorescence microscope.

Isolation of cellular RNA from transfected cells. The procedure used was based on one described elsewhere (54a). Transfections were performed by the calcium phosphate method in 100-mm plates. Forty-eight hours after transfection, the cells were rinsed twice with cold PBS. The cells were scraped in 1 ml of PBS and pelleted in a microcentrifuge (Eppendorf) for 15 s at 1,500 rpm. The cell pellet was resuspended in 450 μl of nuclear lysis buffer (10 mM Tris HCl [pH 8.0], 150 mM NaCl, 1.5 mM $MgCl_2$). Following the addition of 40 μl of 5% Nonidet P-40, the microfuge tubes were inverted once and then incubated for 5 min on ice. The nuclei were then pelleted by centrifugation for 10 to 15 s at 3,000 rpm in a microfuge. The supernatant was transferred to a clean microfuge tube, and 500 μl of phenol-chloroform (1:1) solution and a one-tenth volume of sodium acetate were added. After being briefly vortexed, the samples were centrifuged for 10 to 15 min at 14,000 rpm and the supernatant was ethanol precipitated. The pelleted RNA was resuspended in 100 μl of sterile deionized H_2O and stored at −20°C.

S1 nuclease analysis. The following procedure has been previously described (5). Briefly, RNA isolated from transfected cells was hybridized with a ^{32}P-labeled CAT probe (36) in 20 μl of S1 hybridization buffer {0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.5], 1 mM EDTA} with 80% formamide overnight at 45°C. An S1 nuclease mix containing 1.4 ml of S1 nuclease buffer (0.25 M NaCl, 30 mM sodium acetate [pH 4.6], 1 mM $ZnSO_4$) mixed with 25 μg of heat-denatured, RNase-free, calf thymus DNA and 1,000 U of S1 nuclease (Boehringer Mannheim) was prepared. A 180-μl volume of the S1 nuclease mix was added to each of the hybridized samples. The samples were incubated for 45 min at 30°C, ethanol precipitated, and electrophoresed on a 6% polyacrylamide-urea gel.

Chemical cross-linking with glutaraldehyde. TAX_1 from

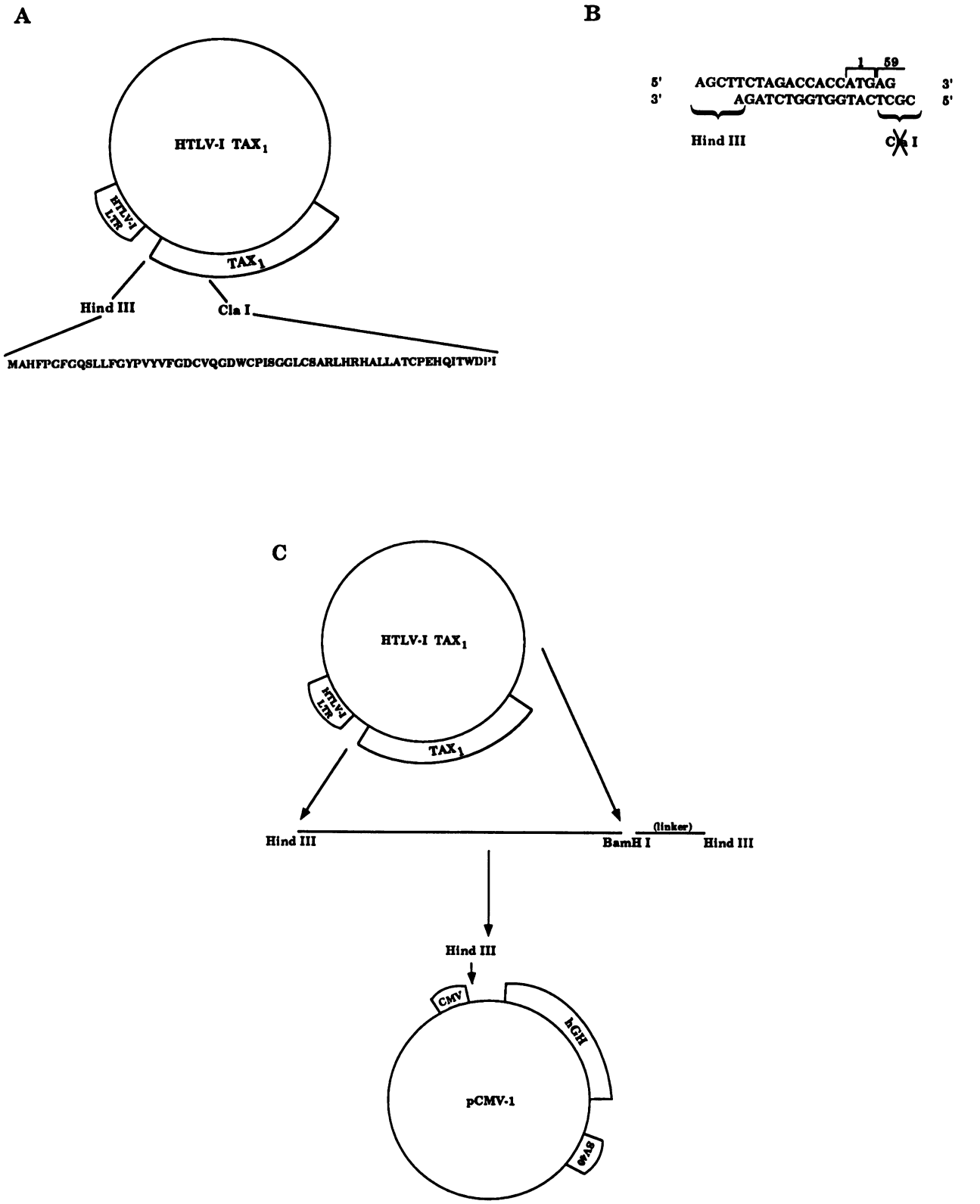

FIG. 1. Construction of the TAX_1 and Δ58 TAX_1 plasmids used. (A) HTLV-I TAX_1 plasmid described previously (41), with the unique *Hin*dIII and *Cla*I sites in the amino terminus of the TAX_1 coding region indicated. The amino acid sequence of the first 59 amino-terminal amino acids residing between the *Hin*dIII and *Cla*I sites is also shown. (B) The synthesized oligonucleotides used to ligate the *Hin*dIII and *Cla*I sites of HTLV-I TAX_1 after the *Hin*dIII-*Cla*I fragment was removed, thus forming HTLV-I Δ58 TAX_1. (C) Construction scheme of the CMV promoter TAX_1 and Δ58 TAX_1 plasmids following removal of the TAX_1 and Δ58 TAX_1 coding regions from the HTLV-I LTR promoter constructs.

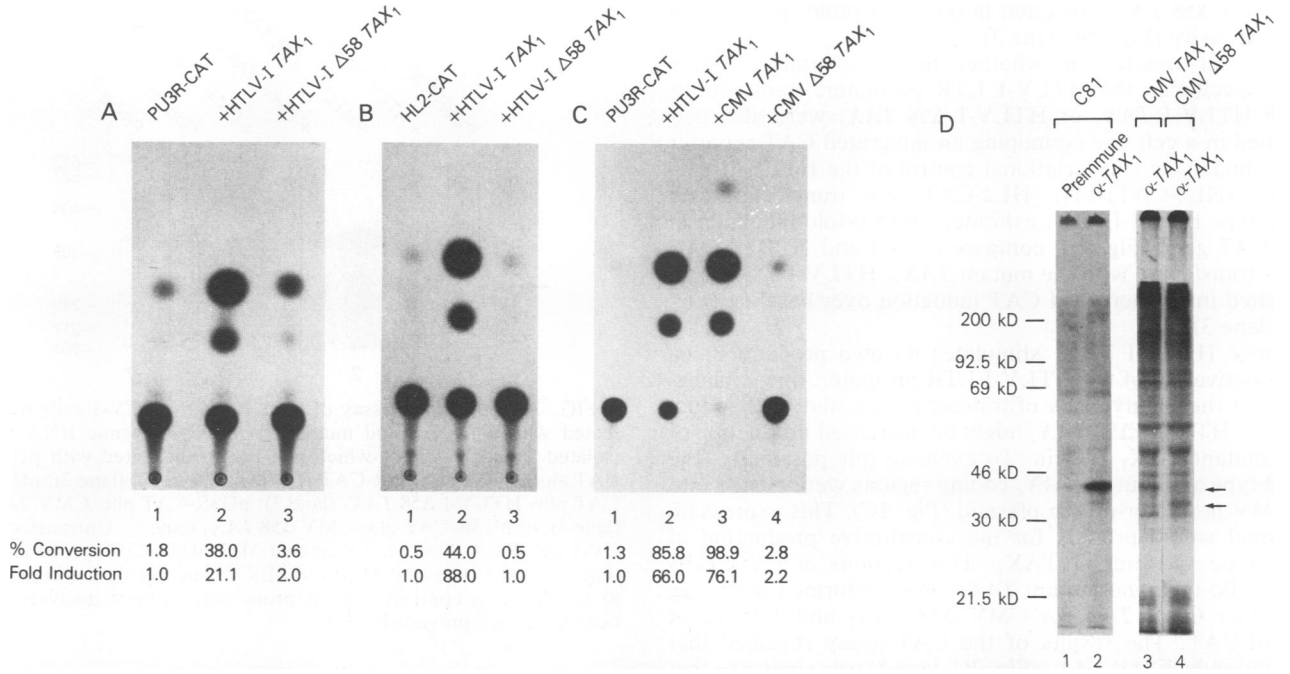

FIG. 2. CAT assays of wild-type and mutant (Δ58 TAX_1) TAX_1 with pU3R-CAT. Cotransfections and CAT assays were performed as described in Materials and Methods. (A) HTLV-I promoter constructs of *TAX*$_1$ and Δ58 *TAX*$_1$ cotransfected with the reporter plasmid pU3R-CAT. A 1-μg amount of HTLV-I *TAX*$_1$ (lane 2) or HTLV-I Δ58 *TAX*$_1$ (lane 3) was cotransfected with 3.75 μg of pU3R-CAT in CV-1 cells. Fold induction of CAT activity was determined by comparison to transfection with pU3R-CAT alone (lane 1). (B) Transfections with 1 μg of either HTLV-I *TAX*$_1$ (lane 2) or HTLV-I Δ58 *TAX*$_1$ (lane 3) in HL2-CAT cells containing an integrated HTLV-II LTR-CAT construct. Fold induction of CAT activity was determined in reference to HL2-CAT cells alone (lane 1). (C) Transfections and CAT assays comparing HTLV-I *TAX*$_1$ (lane 2) with CMV *TAX*$_1$ (lane 3) and CMV Δ58 *TAX*$_1$ (lane 4). A 1-μg amount of the respective *TAX*$_1$ plasmid was cotransfected with 3.75 μg of pU3R-CAT in CV-1 cells. Fold induction of CAT activity was determined in reference to transfection with pU3R-CAT alone (lane 1). (D) Immunoprecipitation and SDS-PAGE analysis of CV-1 cells transfected with 7.5 μg of CMV *TAX*$_1$ (lane 3) or 7.5 μg of CMV Δ58 *TAX*$_1$ (lane 4). Also shown is the analysis of C81 cells (lanes 1 and 2). Cell extracts were immunoprecipitated with either preimmune rabbit serum (lane 1) or polyclonal anti-TAX_1 serum (lanes 2 through 4).

Escherichia coli, which had been purified by ammonium sulfate precipitation, (35a) was dialyzed against approximately 500 volumes of PBS at 4°C prior to chemical cross-linking with glutaraldehyde. Following dialysis, 5 μg of TAX_1 was reacted with 0.01% (final concentration) glutaraldehyde in PBS for 1 or 3 min at 25°C (33). The cross-linking reaction was stopped with the addition of SDS sample buffer (0.1 M Tris HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5% 2-mercaptoethanol), incubated at 90 to 100°C for 5 min, and loaded onto an SDS–10% polyacrylamide gel for electrophoresis. For some samples, 69 μg of the TAX_1 which had been purified by ammonium sulfate precipitation was incubated with 1-, 4-, 10-, or 30-fold molar excess (compared with moles of cysteines in TAX_1) of *N*-ethylmaleimide (24) in PBS at room temperature for 10 min prior to dialysis and cross-linking.

For Western blot (immunoblot) analysis, proteins were electrotransferred to an Immobilon-P (Millipore) membrane for 3 h at 0.3 A (100 to 110 V) with western transfer tank buffer (10 mM Tris HCl [pH 8.2], 0.1 M glycine, 20% methanol) in a Trans-blot unit (Bio-Rad) and then incubated overnight in blocking buffer (2% nonfat dry milk, 10 mM Tris HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA) at room temperature. The membrane was probed with polyclonal rabbit anti-TAX_1 serum in blocking buffer for 3 h. Following five 5-min washes with blocking buffer, the membrane was incubated with 5 μCi of ^{125}I-protein A (Amersham) in blocking buffer for 3 h at room temperature. Following five 5-min washes with milk-free blocking buffer, the membrane was dried and exposed to X-ray film.

RESULTS

Deletion of amino acids 2 through 59 of TAX_1 decreases its transactivational activity. In order to evaluate the functional role of the amino-terminal domain of TAX_1, a deletion mutant of the HTLV-I LTR-driven TAX_1 plasmid, HTLV-I *TAX*$_1$, was made by utilizing the unique *Hin*dIII and *Cla*I restriction sites. After removal of the 186-bp *Hin*dIII-*Cla*I restriction fragment (Fig. 1A), a 20-bp oligonucleotide containing an in-frame initiation codon was ligated into the *Hin*dIII-*Cla*I site (Fig. 1B). This *TAX*$_1$ mutant, HTLV-I Δ58 *TAX*$_1$, was driven by the HTLV-I LTR and, except for the presence of the initiation codon, lacked 58 of the first 59 amino acids.

The ability of the deletion mutant, HTLV-I Δ58 *TAX*$_1$, to transactivate the HTLV-I LTR (pU3R-CAT) was evaluated by performing transient cotransfections in CV-1 cells. Cotransfections were performed by the calcium phosphate method with 3.75 μg of pU3R-CAT alone or with 1 μg of either HTLV-I *TAX*$_1$ or HTLV-I Δ58 *TAX*$_1$. The resultant CAT assays are shown in Fig. 2A. Cotransfection of pU3R-CAT with wild-type HTLV-I *TAX*$_1$ resulted in a 21-fold induction of CAT activity over basal activity (Fig. 2A, lanes 1 and 2). In contrast, cotransfection with the mutant,

HTLV-I Δ58 TAX_1, resulted in only a twofold induction of CAT activity (Fig. 2A, lane 3).

In order to determine whether the loss of transactivation was specific to the HTLV-I LTR promoter, transfections with HTLV-I TAX_1 or HTLV-I Δ58 TAX_1 were also performed in a cell line containing an integrated CAT reporter gene under the transcriptional control of the HTLV-II promoter (HL2-CAT) (44). HL2-CAT cells transfected with wild-type HTLV-I TAX_1 exhibited an 88.0-fold induction of the CAT gene (Fig. 2B, compare lanes 1 and 2). HL2-CAT cells transfected with the mutant TAX_1, HTLV-I Δ58 TAX_1, resulted in no increased CAT induction over baseline (Fig. 2B, lane 3).

Since HTLV-I TAX_1 stimulates its own production via transactivation of the HTLV-I LTR promoter, one explanation for the relative lack of transactivation and CAT induction by HTLV-I Δ58 TAX_1 might be decreased production of the mutant TAX_1 protein. To evaluate this possibility, the wild-type and mutant TAX_1 coding regions were cloned into a CMV promoter-driven plasmid (Fig. 1C). This expression plasmid would provide for the constitutive production of wild-type and mutant TAX_1. Transfections of CV-1 cells with wild-type and mutant TAX_1 were performed with 1 μg of either CMV TAX_1 or CMV Δ58 TAX_1 and 3.75 μg of pU3R-CAT. The results of the CAT assay revealed that induction by CMV TAX_1 (Fig. 2C, lane 3) was similar to that seen with HTLV-I TAX_1 (Fig. 2C, lane 2). In contrast, there was no significant induction of the HTLV-I LTR by CMV Δ58 TAX_1 (Fig. 2C, compare lanes 1 and 4).

To quantitate the synthesis of TAX_1 and Δ58 TAX_1 in the transfected CV-1 cells, immunoprecipitation of ^{35}S-labeled transfected cell extracts with anti-TAX_1 serum was performed. Forty-four hours after transfection, the cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine prior to immunoprecipitation analysis with a polyclonal rabbit anti-TAX_1 serum. The specificity of the immunoprecipitation assay is demonstrated by the C81 cell control (Fig. 2D, lanes 1 and 2). The 40-kDa TAX_1 protein was precipitated with the anti-TAX_1 serum (Fig. 2D, lane 2) but not with preimmune serum (Fig. 2D, lane 1). SDS-PAGE evaluation of the immunoprecipitated proteins from the transfected cells revealed equivalent levels of the wild-type or mutant TAX_1 proteins when the cells were transfected with 7.5 μg of either CMV TAX_1 or CMV Δ58 TAX_1, respectively (Fig. 2D, lanes 3 and 4). In addition, indirect immunofluorescence experiments, described below, also demonstrate that wild-type TAX_1 and Δ58 TAX_1 proteins were expressed equally.

Deletion of TAX_1 amino acids 2 through 59 decreases transcriptional activity as determined by S1 nuclease evaluation of cellular CAT RNA. To demonstrate that the level of CAT expression accurately reflected a change in the steady-state levels of CAT mRNA, RNA was isolated 48 h after transfection and quantitated by S1 nuclease analysis (Fig. 3). The probe used in these studies was synthesized from an M13 vector containing a portion of the CAT coding sequences (36). A protected DNA fragment of 256 nucleotides corresponded to complete protection of the CAT sequences within the probe. Transfections were performed on 100-mm plates and the RNA was isolated as described in Materials and Methods. A 20-μl volume of each RNA (approximately 5 μg) was hybridized with 5 μl (50,000 cpm) of ^{32}P-labeled CAT probe overnight at 45°C, digested with S1 nuclease, and evaluated by electrophoresis on a 6% polyacrylamide-urea gel. Figure 3 shows that the level of CAT RNA is increased in the transfections with wild-type TAX_1 constructs (Fig. 3, lanes 2 and 4) but not in transfections with the mutant, Δ58 TAX_1, constructs (Fig. 3, lanes 3 and 5). Thus, the lack of transactivation that is seen with Δ58 TAX_1 is correlated with the absence of transcription induction of the HTLV-I LTR-containing pU3R-CAT plasmid.

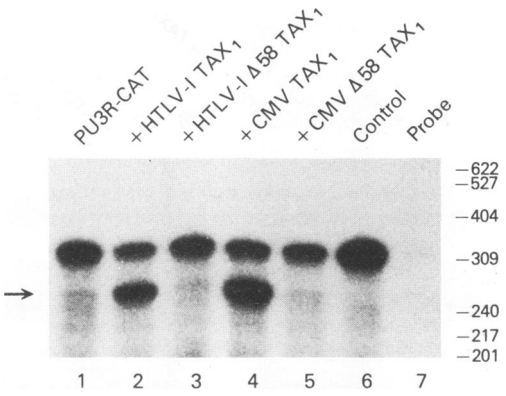

FIG. 3. S1 nuclease assay of CAT RNA from CV-1 cells transfected with wild-type and mutant TAX_1. Cytoplasmic RNA was isolated from CV-1 cells which had been transfected with pU3R-CAT alone (lane 1), pU3R-CAT plus HTLV-I TAX_1 (lane 2), pU3R-CAT plus HTLV-I Δ58 TAX_1 (lane 3), pU3R-CAT plus CMV TAX_1 (lane 4), or pU3R-CAT plus CMV Δ58 TAX_1 (lane 5). Untransfected CV-1 cell RNA (lane 6), ^{32}P-labeled M13 pU3R-CAT probe alone (lane 7), and ^{32}P-labeled *Msp*I-cut PBR322 nucleotide marker (lane 8) are shown as controls. The S1 probe used in these analyses has been described previously (36).

CMV Δ58 TAX_1 is an inhibitory transdominant mutant of TAX_1. In view of the fact that functionally inactive mutant gene products have been found to be inhibitory to the activity of the wild-type gene product, we were interested in determining whether Δ58 TAX_1 had an inhibitory effect on wild-type TAX_1. Cotransfections of 3.75 μg of the HTLV-I LTR-CAT plasmid (pU3R-CAT) with CMV TAX_1 and CMV Δ58 TAX_1 were performed as indicated in the legend for Fig. 4. Cotransfections with increasing amounts of CMV Δ58 TAX_1 (0.125 to 0.75 μg) and a constant amount (1 μg) of CMV TAX_1 led to a significant decrease in CAT activity (Fig. 4, lanes 1 through 4). Wild-type TAX_1 activity was decreased 27.5-fold in the presence of 0.75 μg of CMV Δ58 TAX_1 (Fig. 4, compare lanes 1 and 4). Increasing the amount of CMV Δ58 TAX_1 beyond 0.75 μg resulted in a reproducible but moderate increase in CAT activity (Fig. 4, lanes 5 and 6).

Although the immunoprecipitation assay described above was not sensitive enough to detect wild-type or mutant TAX_1 protein synthesis when the lower amounts of the TAX_1 plasmids were transfected for the CAT assay, cotransfection of 7.5 μg of CMV TAX_1 with 5.6 μg of CMV Δ58 TAX_1 (1:0.75 ratio) did not decrease the synthesis of wild-type TAX_1 protein (data not shown). This suggests that the transdominant effect of Δ58 TAX_1 is not due to an inhibition of the synthesis of the wild-type TAX_1.

Indirect immunofluorescence evaluation of transfections with TAX_1 and Δ58 TAX_1. Transfections of CV-1 cells with TAX_1 and Δ58 TAX_1 plasmids were performed on sterile glass coverslips as described in Materials and Methods. Forty-eight hours after transfection, the cells were fixed onto the coverslips and incubated with rabbit polyclonal anti-TAX_1 serum followed by rhodamine isothiocyanate-conjugated swine anti-rabbit serum. Consistent with cell fractionation studies of TAX_1 in transformed lymphocytes (15, 60,

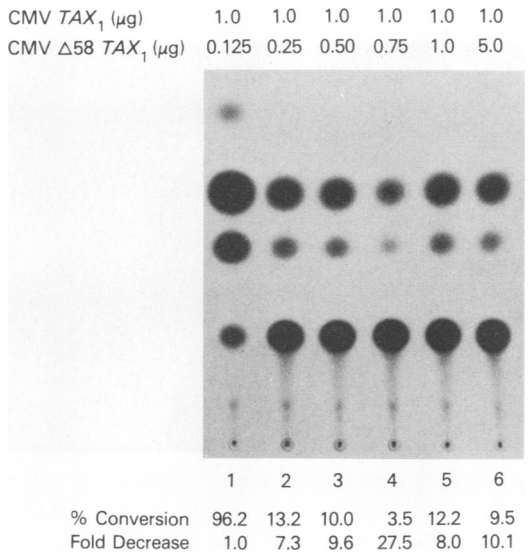

FIG. 4. Transdominant effect of Δ58 TAX_1 on wild-type TAX_1. Transfections were performed with 3.75 μg of pU3R-CAT, 1 μg of CMV TAX_1, and increasing amounts (0.125 to 5.0 μg, as indicated) of CMV Δ58 TAX_1 in CV-1 cells (lanes 1 through 6). Fold decrease in CAT activity was determined in reference to transfection with 3.75 μg of pU3R-CAT and 1 μg of CMV TAX_1 alone (data not shown).

61), wild-type TAX_1 was localized almost exclusively to the nuclei of the transfected CV-1 cells (Fig. 5A). In contrast, however, Δ58 TAX_1 was located in the cytoplasm or perinuclear region of the cell (Fig. 5B). In cotransfections with both CMV TAX_1 and CMV Δ58 TAX_1, TAX_1 was found to localize predominantly in the perinuclear regions of the cells (Fig. 5C and D). Titrating the amount of CMV Δ58 TAX_1 with a constant amount of CMV TAX_1 (1 μg), as done in the experiments demonstrating the transdominant effect of CMV Δ58 TAX_1, revealed that a relatively small amount of TAX_1 can be detected in the nucleus as the amount of CMV Δ58 TAX_1 is increased from 1 to 5 μg (data not shown). Consistent with the inhibition of CAT activity, however, the perinuclear and cytoplasmic localization of TAX_1 predominated over the nuclear localization of TAX_1 in all of these experiments.

Chemical cross-linking of TAX_1 with glutaraldehyde suggests that TAX_1 may function as a multimer. One explanation for the transdominant effect of the Δ58 TAX_1 might be that wild-type TAX_1 forms oligomers with the cytoplasmic Δ58 TAX_1, leading to inefficient transport of TAX_1 to the nucleus of the cell. To investigate this possibility, chemical cross-linking of purified TAX_1 from *E. coli* was performed as described in Materials and Methods. Incubation of the purified TAX_1 (Fig. 6, lane 11) with glutaraldehyde for 1 min revealed distinct TAX_1 species of approximately 40, 80, and 120 kDa by Western blot analysis with a polyclonal anti-TAX_1 serum (Fig. 6, lane 1). Incubating the TAX_1 for longer than 1 min did not increase the relative amount of oligomerized species (Fig. 6, lane 2). Pretreatment of purified TAX_1 with increasing amounts of *N*-ethylmaleimide, an inhibitor of sulfhydryl group (e.g., cysteine) interactions, decreased the relative levels of the 80- and 120-kDa species (Fig. 6, lanes 3 through 10). Similar cross-linking and Western blot analysis of the proteins in a crude whole-cell extract of C81 cells (an HTLV-I-transformed cell line which produces TAX_1) revealed protein complexes of the same size as those shown with the purified TAX_1 (data not shown). These results demonstrate that purified TAX_1 can form homodimers or homotrimers and that multimer formation is dependent upon functional sulfhydryl groups.

DISCUSSION

The role of the amino-terminal domain of TAX_1 in transactivation of the HTLV-I LTR was studied by constructing a plasmid, Δ58 TAX_1, that had amino acids 2 through 59 deleted. CAT assays revealed that Δ58 TAX_1 was unable to transactivate the HTLV-I LTR or the HTLV-II LTR. This ineffective transactivation effect was independent of the promoter controlling the expression of TAX_1, as identical results were obtained with both HTLV-I LTR and CMV promoter-driven wild-type and mutant TAX_1 plasmids. The lack of transactivation by Δ58 TAX_1 was shown by S1 nuclease analysis to be due to the lack of transcription from the HTLV-I LTR. The amino-terminal domain, therefore, appears to be crucial for TAX_1 transactivation of HTLV-I LTR-directed gene expression.

Indirect immunofluorescence demonstrated that the Δ58 TAX_1 failed to localize to the nucleus, which suggests that the TAX_1 nuclear localization signal is located in the first 58 amino acids. Within this sequence, there are no similarities to the Lys-Lys-Lys-Arg-Lys nuclear localization signal of SV40 large T antigen (31, 32) or the Val-Ser-Arg-Pro-Arg signal of polyomavirus large T antigen (48). In fact, the TAX_1 sequence contains little homology to any known nuclear localization signal, including that of adenovirus E1A (37), yeast histone 2B (40), yeast *GAL4* (58), c-*myc* protein (8), heparin-binding (fibroblast) growth factor 1 (26), or HTLV-I REX_1 (59). On the basis of the tendency of nuclear localization sequences to contain a set of basic amino acids, it is interesting to note the basic amino acid sequence Arg-Leu-His-Arg-His (amino acids 38 to 44) in the TAX_1 protein. Introduction of site-specific mutations in this and other amino acids of the amino-terminal domain of the TAX_1 protein will be needed to further define the nuclear localization sequence.

Evaluation of Δ58 TAX_1 by CAT assay and indirect immunofluorescence revealed that Δ58 TAX_1 acts as a transdominant inhibitor of TAX_1 transactivation and nuclear localization. There are several possible mechanisms by which Δ58 TAX_1 might inhibit nuclear localization of TAX_1. As has been postulated in other systems, sequestration of wild-type monomers of a viral regulatory protein into inactive heterodimers might explain the transdominant inhibitory activity (56). By using chemical cross-linking with glutaraldehyde, we have demonstrated that TAX_1 forms multimeric complexes in vitro. The observation that formation of the dimer and trimer forms of TAX_1 is inhibited by the pretreatment of TAX_1 with *N*-ethylmaleimide suggests that sulfhydryl groups are necessary for the heterodimer formation. Four of the eight cysteines present in the TAX_1 protein are in the amino-terminal 58 amino acids. It will be of interest, therefore, to analyze the ability of the Δ58 TAX_1 mutant to form multimeric complexes. On the basis of our present results, we predict that the Δ58 TAX_1 mutant protein would form heterodimer complexes as efficiently as wild-type TAX_1 and that the cysteines at amino acid residues 153, 174, 212, and 261 are important for multimer formation.

Interestingly, a mutant of the HIV-1 TAT basic domain (ΔTAT), which localized to the nucleus and cytoplasm instead of the nucleolus, was transdominant over the wild-

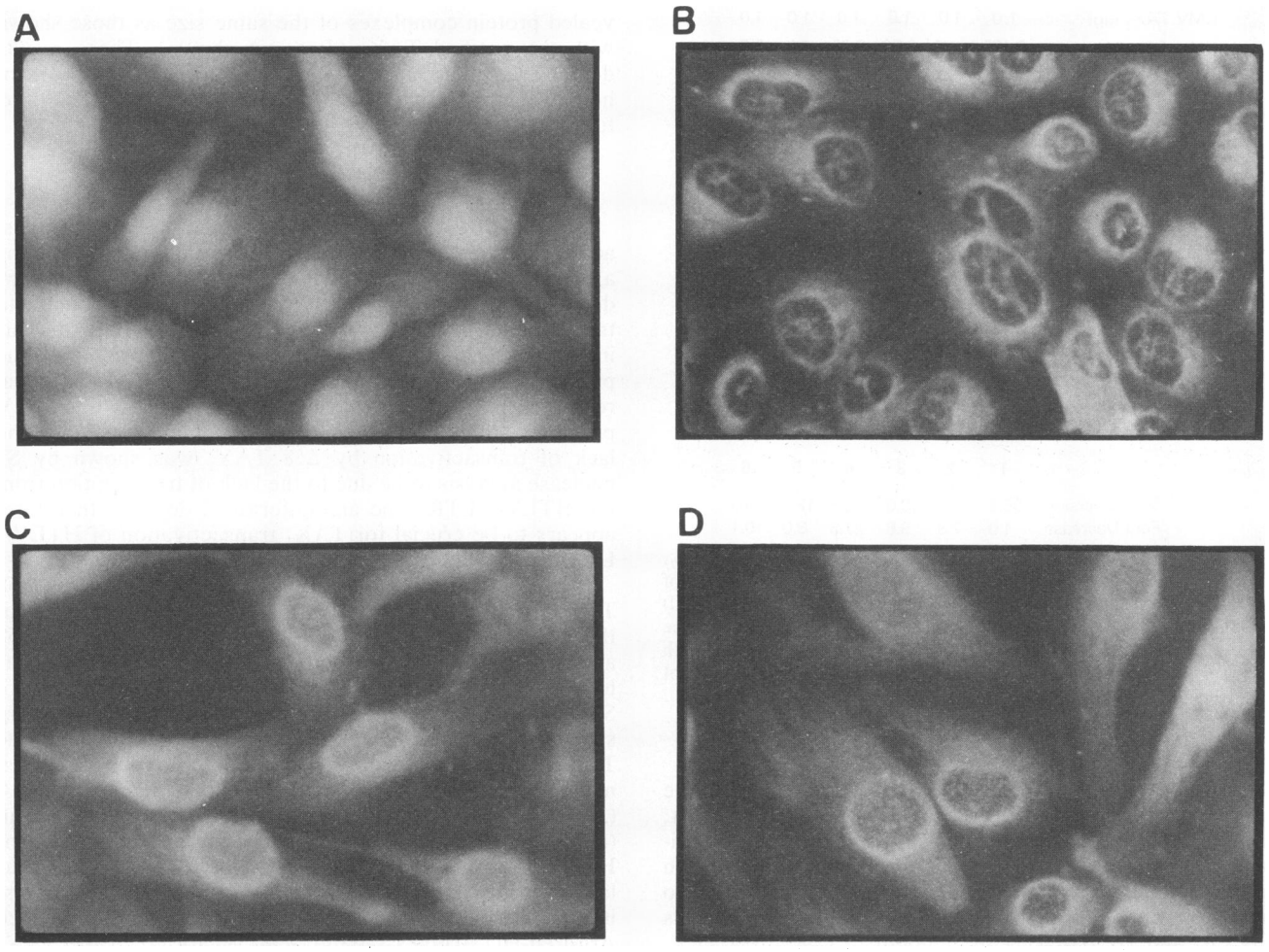

FIG. 5. Indirect immunofluorescence of transfected CV-1 cells with anti-TAX_1 serum. Transfections with CMV *TAX*$_1$ and CMV Δ58 *TAX*$_1$ and preparation of monolayered cells on glass coverslips for indirect immunofluorescence staining were performed as described in Materials and Methods. (A) Transfection with 1 μg of CMV *TAX*$_1$. (B) Transfection with 1 μg of CMV Δ58 *TAX*$_1$. (C) Cotransfection with 1 μg of CMV *TAX*$_1$ and 0.75 μg of CMV Δ58 *TAX*$_1$. (D) Cotransfection with 1 μg of CMV *TAX*$_1$ and 5 μg of CMV Δ58 *TAX*$_1$.

type TAT protein in transactivation assays (45). It is possible that the TAT mutant, by inhibiting the nucleolar localization of the wild-type protein, inhibits the transactivation activity by TAT. Although the mechanism of the nucleolar exclusion remains to be determined, the fact that TAT has been shown to form metal-linked dimers (11) would be consistent with a model in which TAT and ΔTAT form a heterodimer which is defective in nucleolar localization and transactivation.

Our results are consistent with results of the studies of Wachsman et al. (68), who demonstrated that a single amino acid change from proline to leucine at the fifth position of TAX_1 was unable to transactivate the HTLV-I LTR. An identical mutation in HTLV-II TAX_2 reduced the transactivation activity of the protein approximately 30-fold. Interestingly, when the wild-type HTLV-II TAX_2 protein was cotransfected with the mutant HTLV-II TAX_2, an inhibition of HTLV-II LTR transactivation was observed. The authors suggested that the inhibition might be due to competition between the wild-type and mutant gene products for the rate-limiting step in LTR transactivation. In view of the results presented in this report, it would be interesting to determine the cellular localization of these mutants to ascertain whether the amino acid substitution affected the nuclear localization or represented a distinct functional domain of the TAX_2 protein.

The pattern of Δ58 TAX_1 accumulation in the cells is strikingly similar to those described for other proteins which have been shown to be located in the perinuclear region (13) or associated with nuclear pores (9, 42, 47). The transportation of proteins from the cytoplasm into the nucleus appears to involve at least two steps (1, 42, 47). It has been postulated that a protein en route to the nucleus must bind at the nuclear envelope and then be translocated through the nuclear pores. The perinuclear accumulation of Δ58 TAX_1 suggests that binding at the nuclear envelope may be taking place and that the lack of the amino-terminal localization sequence is preventing the translocation of the protein into the nucleus. As more is known about the nuclear transport mechanism, the role and sites of action of nuclear localization sequences should be clarified.

The possibility of a therapeutic role for some of the previously described transdominant mutants has been suggested (21, 38, 45, 49, 66, 67). In this context, for nuclear regulatory proteins, it may be useful to specifically engineer

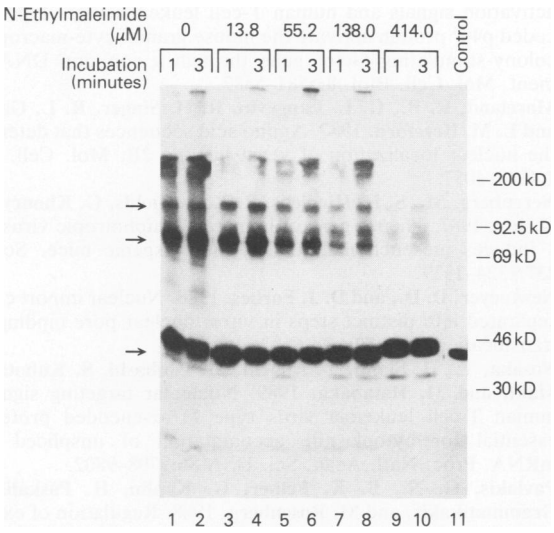

FIG. 6. Chemical cross-linking of TAX_1 with glutaraldehyde. TAX_1 which had been purified by ammonium sulfate precipitation (lane 11) was cross-linked with glutaraldehyde for 1 (lane 1) or 3 (lane 2) min as described in Materials and Methods. Pretreatment of TAX_1 with 1- (lanes 3 and 4), 4- (lanes 5 and 6), 10- (lanes 7 and 8), or 30- (lanes 9 and 10) fold molar excess of *N*-ethylmaleimide was performed prior to glutaraldehyde cross-linking in order to block interactions involving the sulfhydryl groups present. The positions of monomer, dimer, and trimer species of TAX_1 are indicated by arrows.

transdominant mutations in the nuclear localization sequence. Most transforming or transactivating proteins have multiple nuclear targets or functions. For example, the HTLV-I TAX_1 protein regulates expression from several cellular genes including c-*fos* and genes for interleukin-2Rα, interleukin-2, and granulocyte-macrophage colony-stimulating factor. It has been observed that the domains for interleukin-2Rα and HTLV-I LTR transactivation reside in distinct regions of the TAX_1 protein. It is quite possible, therefore, that a transdominant mutant that affects the regulation of one gene has no effect on another gene, making it impossible to create a universal transdominant mutant as long as the regulating protein is targeted to the nucleus of the cell. Sequestration of the regulatory protein to the cytoplasm of the cell, as we have observed with our transdominant HTLV-I TAX_1 mutant, may cirucumvent some of these problems and improve the conceptual approach to the intracellular immunization against essential viral genes.

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ADDENDUM IN PROOF

Following submission of the manuscript, M. R. Smith and W. C. Greene reported that site-directed mutations in the amino-terminal domain of HTLV-I TAX_1 results in the cytoplasmic accumulation of the protein (Genes Dev. **4**:1875–1885, 1990).

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